UPSTREAM TRANSCRIPTIONAL REGULATORY ELEMENTS OF THE *S. CEVEVISIAE CSG2* GENE

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ABSTRACT

Title of Thesis: Upstream Transcriptional Regulatory Elements of the *S. cerevisiae* *CSG2* gene

Sau Ying Lily Wong, Master of Science, 1992

Thesis directed by: Dr. Teresa M. Dunn, Ph.D., Department of Biochemistry

The 5' flanking sequence of *CSG2*, a *Saccharomyces cerevisiae* gene that is required for tolerance of high extracellular calcium, was analyzed to identify transcriptional control elements necessary for its expression. Varying amounts of deletions were created in the 5' flanking region of *CSG2* with nuclease Bal31 treatment of a *CSG2* clone. Deletion plasmids were transformed into yeast and assayed for complementation of the calcium-sensitive growth phenotype of *csg2* null mutant cells. The extent of the deletions was determined by gel electrophoresis and sequencing. The result of this analysis is that only about 30 nucleotides upstream of the presumed start codon of the *CSG2* gene are needed for its expression as assayed by its ability to rescue the calcium-sensitive phenotype of the null mutant strain.

UPSTREAM TRANSCRIPTIONAL REGULATORY ELEMENTS OF THE *S. CEREVISIAE CSG2* GENE

by

Sau Ying Lily Wong

Thesis submitted to the Faculty of the
Department of Biochemistry Graduate Program of the
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DEDICATION

To my mother

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

dNTP (deoxynucleoside triphosphates)

EDTA (ethylenediaminetetraacetic acid)

PEG (polyethylene glycol)

SDS (sodium dodecyl sulfate)

Solution I (50 mM glucose, 25 mM Tris, 10 mM EDTA, pH 8.0)

Solution II (0.12 M sodium hydroxide, 1% SDS)

STET (8% sucrose, 5% Triton X-100, 50mM EDTA, 50 mM Tris, pH 8.0)

TE (10 mM Tris-Cl, pH 7.4, 7.6, or 8.0, 1 mM EDTA)

Tris (Tris(hydroxymethyl)aminomethane)

INTRODUCTION

Calcium metabolism in yeast

Calcium metabolism in eukaryotic cells is a regulated process. Specific transport systems have been shown to regulate the concentrations of calcium inside a cell. Changes in the cytosolic calcium concentration modulate the activity of many protein and enzymes involved in cell function.

Calcium has an important regulatory role in yeast as well. For example, calcium regulates the budding process in yeast (Miyamoto, et al., 1987). Formation of 2 spindle pole bodies during the yeast cell division cycle requires a protein that has homology with calcium binding proteins (Baum, 1986). Calmodulin, a calcium binding protein, must be expressed in yeast for cell proliferation (Ohya and Anraku, 1989). An endopeptidase encoded by the *KEX2* gene is calcium dependent (Mizuno et al., 1989). A calcium ATPase mutation affects yeast secretion (Rudolph et al., 1989). Glucose induces inositolphospholipid metabolism, calcium influx and efflux, and cell proliferation in glucose starved yeast (Kaibuchi et al., 1986; Uno et al., 1988).

Large quantities of calcium can be accumulated by yeast. The amount accumulated depends on the concentration of calcium in the media. For yeast growing in YPD media containing 50 mM calcium, the total cellular calcium concentration is 19 mM (Dunn et al., submitted). The accumulated calcium resides in at least 2 pools. Approximately 10 to 20% of the cellular calcium exchanges with extracellular calcium while the remainder is in a nonexchangeable pool (Eilam, 1982). The function of these calcium pools in yeast is not known.

The cytosolic calcium concentration remains less than 1 μM even when the cells

are placed in media containing 100 mM calcium (Dunn et al., submitted). This accounts for less than 0.01% of the total cellular calcium. To maintain such low cytosolic calcium levels, transporters must exist that sequester the accumulated calcium. Most of the accumulated, nonexchangeable calcium is stored inside the vacuole. An electroneutral calcium/proton exchanger transports calcium into the vacuoles. The vacuolar proton ATPase is responsible for the proton gradient required for the calcium uptake. In cells grown in normal YPD media that has a calcium concentration of 0.28 mM, the free vacuolar calcium concentration is only 20 to 30 μM while the total vacuolar calcium concentration is 2 mM. The explanation for the difference between the free and the total vacuolar calcium concentrations is the binding of the calcium to polyphosphate molecules inside the vacuoles (Dunn et al., submitted).

Another calcium transporter apparently functions in efflux of calcium across the plasma membrane. Efflux of the exchangeable calcium has been shown to be mediated by a transport system that is influenced by extracellular pH. As the extracellular pH drops, the efflux rate goes up (Dunn et al., submitted).

Isolation and analysis of *CSG2*

In order to identify calcium transport systems in *Saccharomyces cerevisiae*, a collection of calcium-sensitive mutants was isolated. Genetic and biochemical analysis of one of the mutants, *csg2*, indicate that it has altered calcium homeostasis. The *CSG2* gene is required for yeast cells to grow at calcium concentrations of 10 mM or higher. A null mutant of the *CSG2* gene demonstrates that the calcium sensitive growth phenotype is due to the lack of a functional, rather than an alteration of, the *CSG2* gene product.

One phenotype of the *csg2* mutant is calcium over-accumulation. The amount of calcium accumulated is more than 10 times the quantity observed for wild-type. Interestingly, the excess calcium accumulated by the *csg2* mutant resides in an exchangeable pool. The cytosolic and vacuolar calcium levels are normal in the mutant. This indicates other organelles in yeast can transport calcium; however, in wild-type cells the amount of calcium in this organelle is small relative to that present in the vacuole.

The wild-type *CSG2* gene was cloned from a yeast genomic library by selecting for plasmids that complement the calcium-sensitive growth phenotype of the *csg2* mutant cells. The complementing activity was mapped to a fragment of DNA containing a 410 amino acid open reading frame. Several transmembrane sequences and a potential calcium binding site of the EF hand type were identified in the proposed amino acid sequence. The data suggest that the *CSG2* gene encodes a membrane protein which either directly mediates or regulates transport of calcium into a nonvacuolar organelle (Beeler et al., submitted).

Yeast upstream transcription control elements

Yeast genes have upstream promoter regions that contain transcriptional control elements. Elements of yeast promoters have been grouped into 4 categories. There are initiation sites, operator sites, TATA elements, and upstream activating sequences (UAS) (reviewed by Struhl, 1989). Initiation sites are places where RNA polymerase II starts transcription. Two consensus sequences for initiation sites that have been identified are TC(G/A)A and purine, purine, pyrimidine, purine, purine (Hahn et al, 1985). Operator sequences are negative control elements usually found upstream of TATA elements. TATA elements are consensus sequences of TATAAA that occur at 40 to 120 base pairs

upstream of the transcription initiation site (Struhl, 1989). Dependent upon the consensus TATAAA sequence, TATA elements have been classified as either being constitutive or regulatory (Struhl, 1989). TATA elements direct where transcription initiation starts in a gene. UAS elements are similar to enhancers except that they only function when located upstream of TATA elements (Guarente, 1987; Struhl, 1989). Their effect on transcription can be positive or negative. They are located upstream of TATA elements at 100 to 1500 base pairs from the initiation site (Struhl, 1989). Figure 1 shows the relative positions of these elements.

Part of the sequence of the *CSG2* gene with its flanking 5' sequence is shown in Figure 2. Some sequences present upstream of *CSG2* that may be important in transcription control are summarized in Table 1. These sequences are located upstream of the proposed start codon for *CSG2*, that is, the first methionine in the 410 amino acid open reading frame thought to encode *CSG2*. The presence of multiple TATA elements and initiation sites has been observed for yeast genes such as *CYC-1*, the yeast iso-1-cytochrome *c* gene. In cases where there are multiple TATA elements and initiation sites, each TATA element orchestrates transcription initiation at more than one initiation site (Hahn et al., 1985). CSG2 also has a stretch of 10 adenines starting at position -145. Adenine and thymine rich sequences have been shown to be important in constitutive expression of genes (Struhl, 1985).

EXPERIMENTAL APPROACH

The parent plasmid, pLW1

The goal of this work was to identify transcriptional control elements in the 5'

Figure 1. General transcription control elements in yeast promoters. From left to right, UAS is upstream activating sequences, OP is operator sequences, TATA is TATA elements, and I is transcription initiation sites (Struhl, 1989). Typical distances between the elements are in the text.

```
-309     *          *          *          *          *
GCCTGCAGAGCAGCGCGCCAAGCAGAAGGAGGCATGGTACTCCTTCTTATTCAAATAAGA
   PstI
-249
TTGTAAATATATGCTACTTTCTTCCTTGTATATACATATATACTTATAACGCTATAACCC

-189
GTTCTTGTAATATCGGCTATCACCCGGCGAAGGTGTATCGCAAGAAAAAAAAAAGCTCTC

-129
TCACTCTAAAGGAGGCTATGTGAACGTGGGAAGATCAAGTGAAAGAAATGTTTGTAGGCC

-69
ATTTTCTTCCAGAACAGATCCGCTCTTGAGCTGGTGAGTTAGCACGATAACAAACAAAGA

-9       +1
TACAGCGTCATGTCTACCACACTACTTTGGTTTTCAAGTGTAATAGGCTACGTGATTCAA
         Met
+52
ACAAAATGTTTGTCTAACATACAATCTAAAAAGGAAATCTCCGTGGGGCCCAATGGTACA
                                                  ApaI
                                              ← Primer
+112
ATTGCAACGCCTGAAACTAACGGCGACAACGGAAACTCAAGTTCATTAACCTTCTATCTG

+172
ACCTTTATGTATTTTGCTTCGTGGCTGCTCTTGGTGCCTGCATCTCGACTTTGGGAGAAG
      Met
+232
ATGAGACCGATGTTTGTCTCTGACTCAGACTCGAACAGGAATTCTCAGTT
                                          EcoRI
```

Figure 2. Upstream, 5' flanking sequence, and part of the sequence for the *CSG2* gene. This shows the *PstI* to *EcoRI* fragment of *CSG2*. The asterisks on top mark every 10 nucleotides. The numbering gives positions of bases at extreme left of lines, and is shown relative to the first underlined ATG codon. Useful restriction enzyme sites are underlined and labeled. The first putative start codon, ATG, is underlined and labeled as well as another methionine further downstream. The site for the *CSG2* specific primer used in double stranded sequencing is also underlined and labeled.

Table 1. *CSG2* sequences with identity to yeast promoter consensus sequences.

Consensus Element	*CSG2* Sequence	Position
TATA	TATA	-242
	TATAA	-205, -197
	TATATA	-221, -213
Transcription Initiation	TCAA	-95
	GGCGA	-164
	AGTGA	-92
	AACAG	-57
	GGTGA	-37
	GATAA	-24
	AACAA	-21, -17

The positions indicated are relative to the first putative start codon found in DNA cloned for *CSG2* to the first nucleotide of the consensus sequence (see Figure 2). The consensus sequences for the TATA elements are based on the variations of TATAAA. The sequence of TATATA has been shown to be a regulatory TATA element in the *HIS3* promoter (Struhl, 1989). The transcriptional initiation consensus sequences are from Hahn et al. (Hahn et al., 1985; Guarente, 1987).

flanking region of the *CSG2* gene as part of the laboratory's characterization of *CSG2*. To approach this, we subcloned the *CSG2* gene with flanking 5' and 3' sequences into a shuttle vector, pRS314. This *CSG2* clone, pLW1, was used for the generation of a set of nested deletions at the 5' end. The general properties of pLW1 is shown in Figure 3.

Generation and analysis of deletion plasmids

The 5' deletions were generated by cutting pLW1 at the *PstI* site and treating with nuclease Bal31 for different times. After linearizing pLW1 at the 5' end of the *CSG2* insert with *PstI*, nuclease Bal31 removed nucleotides from both ends. The Bal31 treated DNA was repaired with dNTPs and the Klenow Fragment. Linkers with the site for restriction enzyme *SalI* were ligated to the repaired ends. The families of deleted pLW1 plasmids were transformed into and amplified from bacteria in large scale plasmid preps.

DNA purified from the large scale plasmid preps was digested with *SalI* and *EcoRI* to generate a family of fragments extending from the deletion endpoint to codon 91 of the proposed *CSG2* protein. The *SalI* and *EcoRI* digested DNA was electrophoresed and the *SalI* to *EcoRI* deletion bands were purified from the gel. The experimental approach used is summarized in Figure 4.

In order to assure that the sequence flanking the 5' deletion end points is constant for each deletion mutant, the DNA fragments from the sets of nested deletions described above were subcloned into constant vector #4. Constant vector #4, c.v.#4, differs from pLW1 only in that a small amount of sequence at the *PstI* site was removed with Bal31 and a *SalI* linker was inserted. Figures 5 and 6 show the construct of constant vector #4 and the deletion plasmids respectively. This subcloning was done to provide a suitable

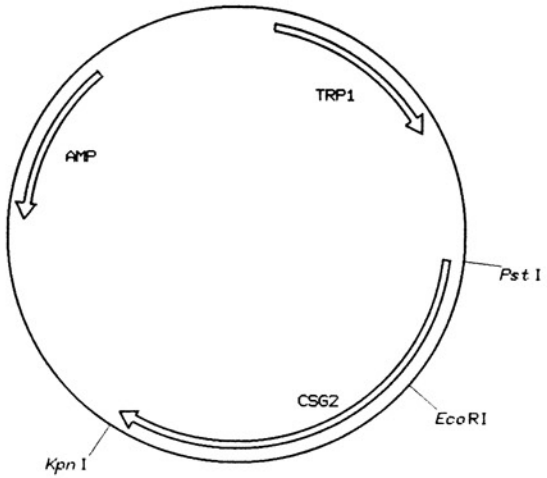

Figure 3. General properties of the parent plasmid, pLW1. The *AMP* and *TRP1* genes are for ampicillin resistance in bacteria and tryptophan metabolism in yeast, respectively. Useful restriction enzyme sites in the *CSG2* gene are indicated.

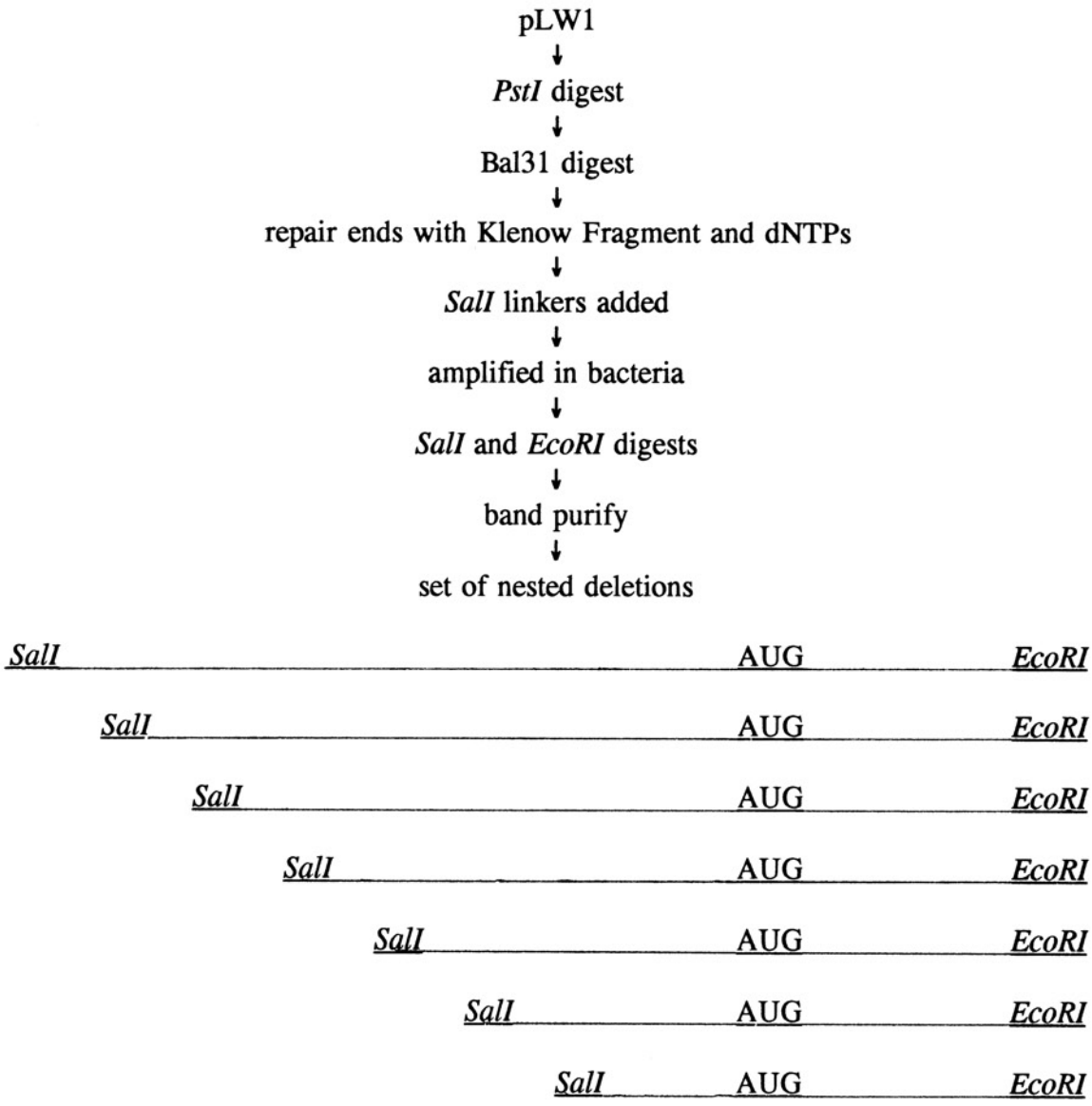

Figure 4. Experimental approach for generating nested deletions. Details for each step are in the Goals and Methods sections. As indicated the *SalI* sites are the varying end points in the 5' flanking sequence of the *CSG2* gene, and the *EcoRI* sites are the constant point in codon 91 of *CSG2*.

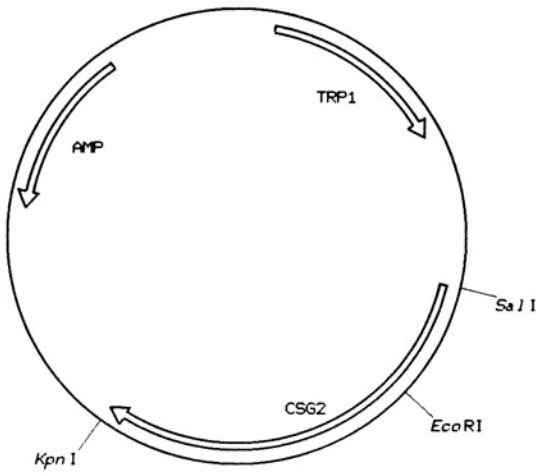

Figure 5. General properties of the constant vector #4, c.v.#4. The *CSG2* gene's upstream region is shorter than in pLW1, and a *SalI* site replaced the original *PstI* site.

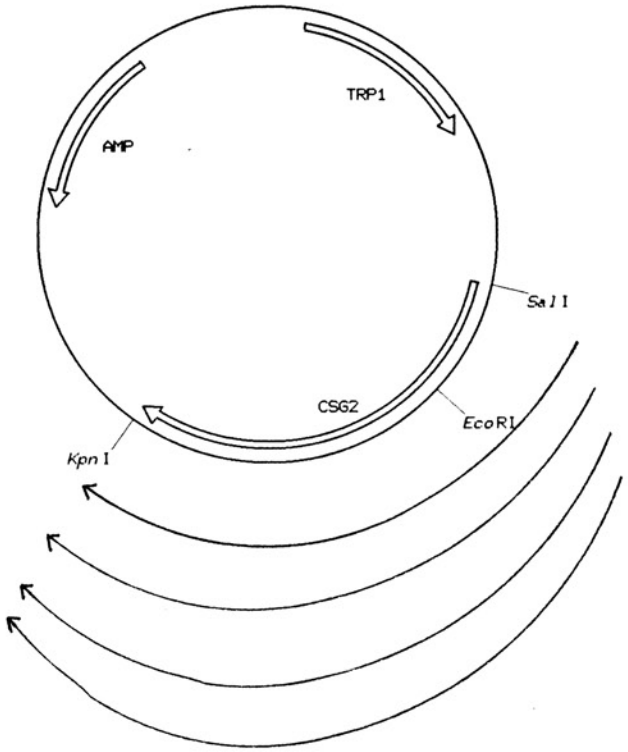

Figure 6. General properties of the deletion plasmids. The arrows represent the various deletions in the 5' flanking sequence of the *CSG2* gene.

vector into which the *SalI* to *EcoRI* deletion fragments could be placed to prevent potential influence of variable upstream vector sequences on expression of the *CSG2* gene.

The deletion plasmids were analyzed by a combination of assays. First they were analyzed for the extent of the deletion using gel electrophoresis. Based on these results, some were selected for the complementation assay and sequencing.

MATERIALS

Chemicals

All chemicals used were of the appropriate grade identified by the procedure.

Cells

Competent bacterial cells, Epicurian Coli AG1 (*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* (*rk-*, *mk+*), *supE44*, *relA1*) and Epicurian Coli SCS1 (derived from Epicurian Coli AG1), were purchased from Stratagene.

Yeast competent cells, TDY2039 (a *CSG2*$^+$ *ade* 2-101 *LYS*$^+$ *ura* 3-52 *trp* 1 Δ *leu* 2 Δ) and TDY2040 (a *csg2*::*LEU2*$^+$ *ade* 2-101 *LYS*$^+$ *ura* 3-52 *trp* 1 Δ*leu* 2 Δ), were prepared as described in the methods section.

Recombinant DNA material

DNA modifying enzymes and restriction enzymes were purchased from Bethesda Research Laboratories or New England BioLabs. Linkers for the restriction enzyme SalI, d(CGGTCGACCG), were purchased from Stratagene. Shuttle vectors, pRS314 and pRS316, were from Sikorski and Hieter (1989); YCp50 vectors were from Botstein et al. (1979). Deoxynucleoside triphosphates were purchased from Pharmacia. Sequencing kits for double stranded DNA were purchased from Applied BioSystems. The primer,

dCTCCGTGGGGCCCAATGG, used for the double stranded DNA sequencing was prepared by Mike Flora of the USUHS Oligonucleotide Synthesizing Facility.

METHODS

Recombinant plasmid constructions

A *PstI* to *KpnI* fragment that contains the *CSG2* gene was ligated to pRS314 digested with the same restriction enzymes. Restriction enzymes were used according to the supplier. They were inactivated by phenol extraction. Ligation reactions were done at 12°C for 4 hours or overnight. T4 DNA ligase was inactivated by phenol extraction or by incubating at 65°C for at least 20 minutes. The ligation reaction was transformed into Epicurian Coli AG1 or SCS1. DNA was prepared by a modification of the boiling mini plasmid prep procedure of Holmes and Quigley (1981) described below, and candidates were subjected to restriction mapping to ascertain that the desired construct had been generated. Once pLW1 was constructed, a large scale plasmid prep was done as described by Maniatis et al. (1982).

The collection of nested deletions was created by treating *PstI* linearized pLW1 with Bal31 as described by Davis et al. (1986), except aliquots of the reaction were removed at 0.5, 1, 2, and 4 minutes to tubes containing EDTA. The DNA was phenol extracted, ethanol precipitated, and the extent of deletion was assessed by agarose gel electrophoresis. The digested ends were filled in with dNTPs using Klenow Fragment, *SalI* linkers were ligated on, and the ligation was transformed into *E. coli*. The transformants for each time point were pooled by washing the plates with LB media and the wash was used as inoculant for large scale plasmid preps. The expectant *SalI* to *EcoRI* fragments were isolated from the DNA of the large scale plasmid preps by using

membrane strips as described below.

The *SalI* to *EcoRI* fragments were ligated to constant vector #4 to create deletion plasmids. They were named Δ pLW1S#P#. The Δ pLW1 designates that they are derived from pLW1, the S specifies that a *SalI* site is placed at the deletion junction, the first number is the time point from the Bal31 deletions, the P indicates the modified site was originally a *PstI* site of pLW1 and the last number specifies the different plasmids for the time point.

Media

LB and LB ampicillin at 50 μg/ml media and plates were used for growing bacteria and were made according to standard procedures. SOC media was used in transformation of bacteria and was made according to Stratagene. YPD and SD media were used for yeast and were made according to Sherman (1991).

Plasmid preps

Rapid plasmid preps were done as described by Holmes and Quigley (1981) from transformed *E. coli* cells streaked onto LB ampicillin plates and incubated at 37° C overnight. Cells were scraped into 0.7 ml of STET and resuspended. To each sample, 25 μl of freshly made 10 mg/ml lysozyme in STET was added, vortexed, incubated at room temperature for 10 minutes, incubated at 90°C for 90 seconds, incubated at room temperature again for 10 minutes, and centrifuged at 4°C at 14K rpm for 30 minutes. The supernatant was transferred to a new tube, an equal volume of isopropanol was added, vortexed, and centrifuged at 4°C at 14K for 5 minutes. Pellets were resuspended in 200 μl of TE, mixed well with 80 μl of 7.5 M ammonium acetate, incubated on ice for 15 minutes, and centrifuged at 4°C at 14K rpm for 5 minutes. The supernatant was

transferred to a new tube, two volumes of cold (-20°) ethanol was added, incubated on ice for 10 minutes, and centrifuged at 4°C at 14K rpm for 5 to 10 minutes. Ethanol was removed and each pellet was resuspended with 100 μl of 2X TE with 20 μg/ml RNaseA, and incubated at 37°C for at least 15 minutes. Each sample was phenol extracted with 100 μl of phenol; the aqueous layer was transferred to a fresh tube, 100 μl of 0.6 M sodium acetate and 600 μl of cold ,-20°C, ethanol was added, and the mixture incubated at -20°C for at least 30 minutes. After centrifugation at room temperature at 14K rpm for at least 10 minutes, each pellet was air dried and resuspended with 100 μl of TE. Typical yields from these plasmid preps were 10 to 20 μg DNA.

Large scale plasmid preps were done as described by Maniatis et al. (1982) with 500 ml cultures of transformed Epicurian Coli AGI cells. They were grown at 37°C with shaking in LB ampicillin to an OD_{600} of 0.4 at which time 2.5 ml of 34 mg/ml chloramphenicol in ethanol was added. Cells were incubated at 37°C with shaking overnight. They were harvested by centrifugation at 4K rpm for 10 minutes, pellets were washed with about 20 ml of TE and repelleted. Pellets were resuspended in 4 ml of solution I, 4 ml of solution I with 10 mg/ml lysozyme added, vortexed, incubated at room temperature for 5 minutes. Sixteen ml of freshly prepared 0.12 mM sodium hydroxide and 1% SDS was added, mixed by gently inverting the tube, and incubated on ice for 10 minutes. Finally, 12 ml of 5 M potassium acetate was added, the tube was inverted 10 times, allowed to incubate on ice for 10 minutes, and centrifuged at 4°C at 17K rpm for 30 minutes. The supernatant was transferred to 12 ml of isopropanol, incubated at room temperature for 15 minutes, and centrifuged at room temperature at 7K rpm for 10 minutes. Pellets were resuspended in 2.5 ml of 2X TE, about 1/3 volume of 7.5 M ammonium acetate was added, tubes were vortexed, and incubated on ice for

15 minutes, followed by centrifugation at 4°C at 8K rpm for 10 minutes. The supernatant was transferred to 15 ml of cold ethanol, incubated on ice for 15 minutes, and centrifuged at 4°C at 8K for 10 minutes. Pellets were resuspended in 10 ml of 2X TE with 20 μg/ml RNaseA and incubated at 37°C for 20 minutes. Five ml of 24% PEG 8000, 1.5 M sodium chloride, 0.1 M Tris, pH 8, 5 mM EDTA was added. After incubating on ice for 2 hours, the samples were centrifuged at 4°C at 8K rpm for 10 minutes. Pellets were resuspended in 5 ml of TE and ethanol precipitated with 0.5 ml of 3 M sodium acetate and 12 ml of cold ethanol with incubation at -20°C for at least 30 minutes. Samples were centrifuged at 4°C at 8K rpm for 10 minutes. Pellets were resuspended with 200 μl of 0.5X TE and stored at -20°C. Yield was determined by electrophoresing 2 μl of each sample in a 1% agarose gel and estimated relative to concentration standards. Typical yields were from 175 to 300 μg DNA.

Gel electrophoresis analysis

Gel electrophoresis, both agarose and polyacrylamide, was done according to standard procedures (Schleif and Wensink, 1981). Agarose gels were used analytically for determining yield of plasmid preps, band isolations, and restriction enzyme digests as well as preparatively for band isolations. Polyacrylamide gels were used analytically for sizing some of the deletion fragments.

Analysis of restriction enzyme digests or yields from DNA preps were usually done on 1% agarose gels containing 0.5 μg/ml of ethidium bromide in both the gel and running buffer. For analysis of some of the deletion fragments, 1.8% agarose, 5%, and 10% polyacrylamide gel electrophoresis were also used. The polyacrylamide gels were poured from 20% acrylamide and 0.66% methylene bisacrylamide solutions and stained with ethidium bromide after electrophoresis. Molecular weights for DNA were estimated

by comparison with standards run alongside the samples. Sizes were estimated manually form standard curves or with a computer program, Frag Gel (written and kindly provided by Kenneth S. Gable).

DNA fragment isolation

DNA band isolations were done when specific DNA fragments were needed for subcloning. The procedure is a modified version of one described by Maniatis et al. (1982). Restriction enzyme digested DNA was electrophoresed in a 1% agarose gel containing 0.5 μg/ml of ethidium bromide. Long wave UV light was used to visualize the DNA bands. Slits were cut in the gel above and below the bands of interest, NA45 membrane strips soaked in TE were inserted into the slits, and electrophoresis was continued until the band had run into the strips. The strips were then removed and rinsed in TE. The DNA fragments were eluted by incubating in 250 μl of 1 M sodium chloride, 50 mM arginine, and 10 mM Tris, pH 8, at 65°C for up to 30 minutes with flicking about every 5 minutes. The strip was removed, 2 μl of tRNA at 10 mg/ml was added, and the solution was phenol extracted. The aqueous phase was ethanol precipitated with 600 μl of cold ethanol at -20°C for at least 30 minutes. Pellets from centrifugation at room temperature at 14K rpm for at least 10 minutes were resuspended with 75 μl of 0.3 M sodium acetate, pH 6, and ethanol precipitated again with 200 μl of cold ethanol at -20°C for at least 30 minutes. After centrifuging the samples as before, the pellets were resuspended in 25 μl of TE. An aliquot, usually 5 μl, was electrophoresed in a 1% agarose gel along with molecular weight standards to determine yield and purity. When the carrier tRNA comigrated with purified DNA fragment, it was necessary to treat with 1 μl of 10 mg/ml RNase A at 37°C for at least 20 minutes before electrophoresis.

Transformation into bacteria

Transformation into Epicurian Coli cells was done as per procedures that accompanied the competent cells purchased from Stratagene. On ice, 1.7 to .85 μl of 1.4 M ß-mercaptoethanol was added to 100 to 50 μl of competent AG1 cells and left for 10 minutes with swirling every 2 minutes. DNA was added, typically in the range of 0.1 to 50 ng, and incubated on ice for 30 minutes. The transformation reactions were heat shocked at 42°C for 45 seconds and placed back on ice for 2 minutes. To each transformation reaction, 0.9 ml of SOC media at 42°C was added. They were then incubated at 37°C for 1 hour. Aliquots of the transformation reaction were spread onto LB ampicillin plates and incubated at 37° overnight.

Transformation into yeast

Yeast transformations were done using either the lithium acetate procedure of Ito et al. (1983) or the electroporation procedure described by Becker and Guarente (1991). For the lithium acetate procedure, yeast cells were grown at 26°C with shaking to an OD_{600} of 0.8 to 1.0. They were harvested and washed with water. Pellets were resuspended in a solution of 0.1 M lithium acetate, 10 mM Tris, 1 mM EDTA, and 15% glycerol, pH 7.5 at 1/200 of the volume the yeast cells were grown in. Aliquots were frozen in an ethanol and dry ice bath and stored at -80°C.

For each transformation reaction, 100 μl of competent yeast cells were used. After defrosting on ice, they were incubated at 26°C with shaking for 0.5 to 1 hour. The recombinant DNA and sonicated salmon sperm DNA were then added, usually at least 1 μg and 6 μg of each respectively and incubated at 26°C for 5 to 30 minutes. One ml of 40% PEG 4000, 0.1 M lithium acetate, 10 mM Tris, pH 7.5, and 1 mM EDTA was added to each of the samples. They were incubated at 26°C for 0.5 to 1 hour, heat

shocked at 42°C for 5 minutes, pelleted, washed once with water, resuspended with 500 μl of water, spread onto selective plates, and incubated at 26°C for about 3 days.

Transformations into yeast were also done with electroporation as described by Becker and Guarente (1991). Yeast cells were grown to an OD_{600} of 1.3 to 1.5. They were washed twice with cold distilled water, first with the original volume of the culture and second with half of the original volume. Twenty ml of cold 1 M sorbitol per 500 ml of original culture was used to wash the cells. Pellets were resuspended with cold 1 M sorbitol, 1 ml per 500 ml original volume of the culture. Eighty μl of the yeast cells were aliquoted for each transformation. About 100 ng of DNA was added per transformation. The cells and DNA mixture was transferred to a cold Bio-Rad Laboratories 0.2 cm electrode gap gene pulser cuvette and pulsed at 1.5 kV, 25 μF, 200 Ω with a Bio-Rad Gene Pulser with Bio-Rad Pulse Controller. One ml of cold 1 M sorbitol was added after the pulse, mixed, and 200 μl of the transformation was spread on selective plates containing 1 M sorbitol. Plates were incubated at 26°C for at least 3 days. Typical transformation efficiencies by the electroporation method range from 10^3 to 10^4 colonies per μg of DNA.

Complementation assay

The yeast strain TDY2040 is a null mutant of the *CSG2* gene (Beeler et al., submitted). It will grow only in high calcium concentrations if it contains an extrachromosomal plasmid and can express a functional *CSG2* gene product from the plasmid. TDY2040 cells transformed with the appropriate plasmid were first selected for tryptophan prototrophy conferred by the *TRP1* gene of the vector; tryptophan prototrophs were then streaked onto selective plates with or without calcium. Two calcium concentrations were used in the selective plates, 100 mM and 200 mM. The

plates were incubated at 26°C or 37°C for at least 3 days before scoring for growth.

DNA sequencing

Double stranded templates were prepared using a plasmid kit purchased from Qiagen. Alternatively, DNA already prepared by our laboratory's standard rapid plasmid prep was purified with the Qiagen column prior to sequencing. *CSG2* specific primers were synthesized by Mike Flora of the USUHS Oligonucleotide Synthesizing Facility.

DNA for double stranded sequencing was prepared as per instructions accompanying the Qiagen Plasmid Kit. Up to 5 ml of an overnight culture was pelleted. The pellets were resuspended in 0.3 to 0.6 ml of 50 mM Tris, 10 mM EDTA, pH 8 with 100 μg/ml RNaseA. A volume of 0.3 to 0.6 ml of a solution of 200 mM sodium hydroxide, 1% SDS was added, the tube inverted several times, incubated for 5 minutes at room temperature, 0.3 to 0.6 ml of 2.55 M potassium acetate, pH 4.8 was added, inverted several times, centrifuged at 4°C at 14K rpm for 15 minutes, and the supernatant was applied to an equilibrated column. Two ml of the wash buffer, 1 M sodium chloride, 50 mM MOPS, 15% ethanol, pH 7, was used to wash the column. To elute the DNA samples, 0.8 ml of the elution buffer, 1.25 M sodium chloride, 50 mM MOPS, 15% ethanol, pH 8.2, was allowed to run through the column and any remaining liquid was forced out. One-half volume of room temperature isopropanol was added, mixed, and the sample was centrifuged for 30 minutes. The pellet was dried and resuspended in the desired volume of water.

To further purify DNA from rapid plasmid preps for sequencing, the DNA samples were diluted 10-fold with equilibration buffer, 750 mM sodium chloride, 50 mM MOPS, 15% ethanol, pH 7 and the Qiagen Plasmid Kit protocol followed from the point of equilibrating the column.

The Taq DyeDeoxy Terminator Cycle Sequencing Kit from Applied Biosystems was used for double stranded sequencing. Samples were given to Stephen T. White for electrophoresis and collection of sequences with an automated sequencer, Applied Biosystems Model 373A DNA Sequencer. Sequences were analyzed manually or on a computer with Microgenie: Sequence Analysis Program from Beckman.

RESULTS

Generation and properties of the deletion mutant collection

Nested deletions from the *PstI* site into the 5' flanking region of the *CSG2* gene were created as described in Figure 4 as well as the goals and methods sections. There were approximately 3100, 2500, 1200, and 2000 transformants from 0.5, 1, 2, and 4 minute time points respectively from the transformation of the Bal31 digested, blunt ended, and *SalI* linkered pLW1. These transformants served as inoculant for large scale plasmid preps of the modified pLW1. Subsequent *SalI* and *EcoRI* digestions and band isolation of the desired fragments from these large scale plasmid preps yielded the sets of nested deletion mutants. The size of these fragments in base pairs were in the range of about 400 to 600 for 0.5 minute, 300 to 550 for 1 minute, 200 to 400 for 2 and 4 minutes of incubation with nuclease Bal31.

As described in the experimental approach section, the DNA fragments that contain the nested deletions in the 5' flanking sequence of *CSG2* were subcloned into c.v.#4. This subcloning step was done to insure that adjoining sequences between pRS314 and the deleted *CSG2* 5' flanking end is constant in each deletion mutant. The amount of 5' flanking sequence each deletion plasmid contained was sized by gel electrophoresis. Figure 7 shows some of the deletion fragments that were analyzed on

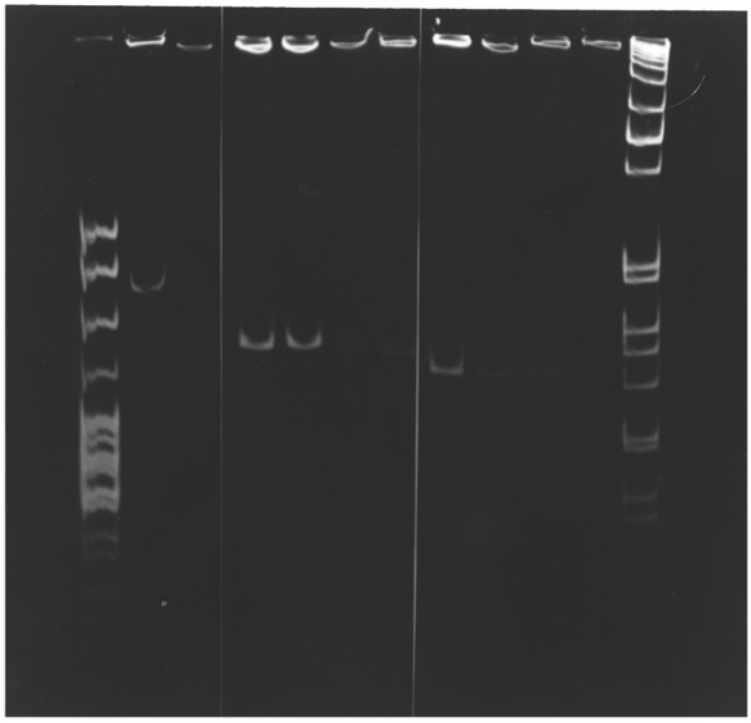

Figure 7. Some members of the set of nested deletions. The deletion plasmids were digested with *SalI* and *EcoRI* and electrophoresed in a 5% polyacrylamide gel. In the extreme left and right lanes are molecular weight standards. From left to right, the deletion plasmids are: constant vector #4, Δ pLW1S30sP#7, Δ pLW1S2'P#13, Δ pLW1S30sP#40, Δ pLW1S2'P#4, Δ pLW1S2'P#5, Δ pLW1S1'P#38, Δ pLW1S2'P#9, Δ pLW1S2'P#46, and Δ pLW1S1'P#5.

a 5% polyacrylamide gel. Each deletion plasmid was digested with *SalI* and *EcoRI*, at the varying 5' deletion end point and the constant site within *CSG2*'s coding region respectively.

Based on the approximate size of the deletion at the 5' flanking region of *CSG2*, several of the deletion plasmids were assayed for their ability to complement the calcium-sensitive growth phenotype of the *csg2* null mutant. Figures 8a, 8b, 8c shows the positive results of the complementation assay for c.v.#4 as compared to the negative results for pRS314, the vector without any *CSG2*. The exact amount deleted at the 5' flanking region of the *CSG2* deletion plasmids was determined by sequencing. The results are presented in Table 2. Because the complementation assay only gives results of growth or no growth, there is no indication for weak complementation except slow growth. There is one such weak complementation plasmid in Table 2, Δ pLW1S2'P#46. Cells transformed with this plasmid grow slower than cells transformed with deletion plasmids containing longer 5' flanking sequence. Figure 9 summarizes the complementation results of the deletion plasmids in Table 2 in the 5' flanking sequence of *CSG2*. The clear break in the data between the complementing and noncomplementing deletion plasmids suggest that only about 30 nucleotides upstream of the proposed start codon for the 410 amino acid open reading frame are necessary for expression of the *CSG2* gene.

The sequence flanking the deletion endpoints are not constant

Sequence analysis also lead to the unexpected observation that the pRS314 vector sequence adjacent to the end points of the deleted 5' flanking sequence of *CSG2* is not constant. The pRS314 3'end point of the *CSG2* clones is given in the last row of Table 2. The size of the variable region is 400 nucleotides between the least to the most

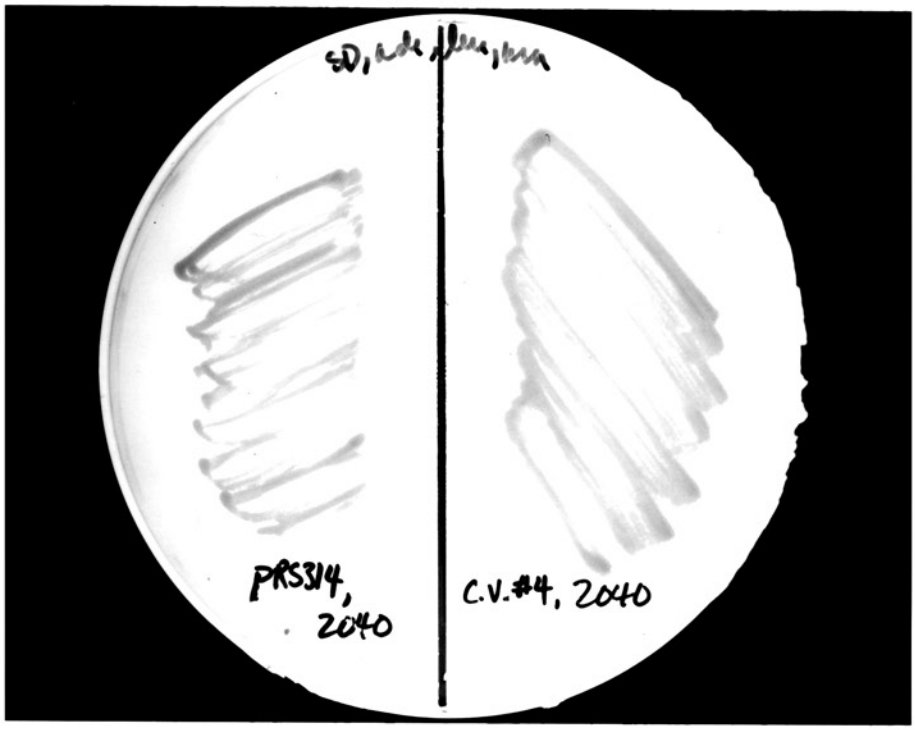

Figure 8a. Sample of complementation assay with selective plates. Transformed TDY2040 yeast cells, null mutants for the *CSG2* gene, are streaked onto a minimal media plate supplemented with adenine, leucine, and uracil. The transformed plasmids on the right and left are c.v. #4 and the shuttle vector, pRS314, respectively.

Figure 8b. Sample of complementation assay with 100 mM calcium plates. The plate is as described for Figure 8a with 100 mM calcium added. The streaked cells are from the same inoculant as Figure 8a.

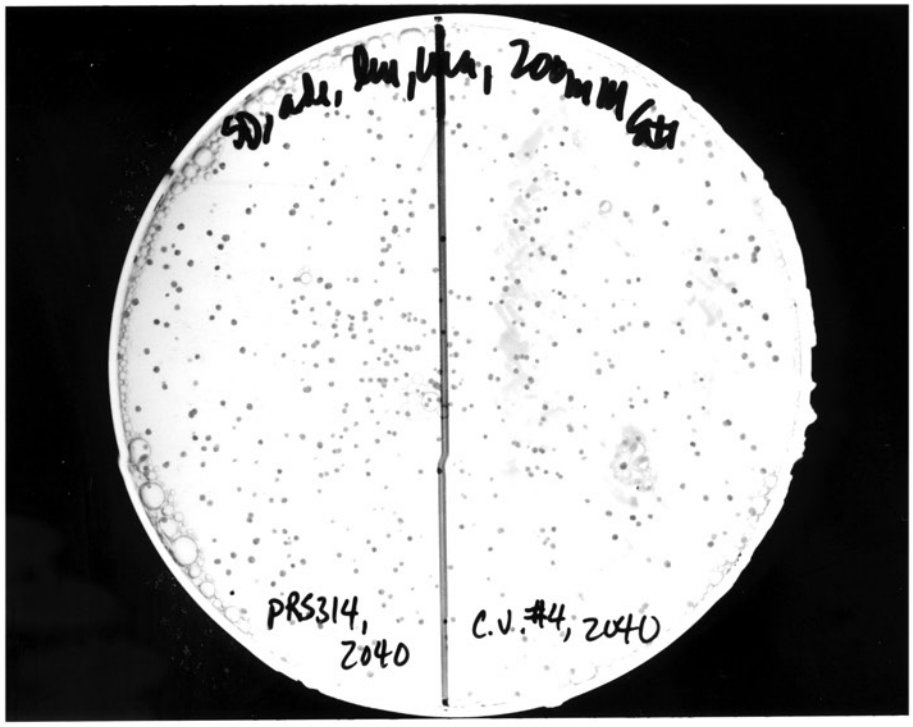

Figure 8c. Sample of complementation assay with 200 mM calcium plates. Streaks and plates are the same as for Figure 8b, except the plate has 200 mM calcium.

Table 2. Summary of some members from the family of deletion plasmids.

Deletion Plasmid	Complementation	*SalI-EcoRI* Fragment (bp)	pRS314 End Point
Δ pLW1S30sP#7	+	444	1835
Δ pLW1S30sP#63	+	439	1616
Δ pLW1S30sP#34	+	433	1848
Δ pLW1S30sP#47	+	388	1897
Δ pLW1S1'P#55	+	343	1616
Δ pLW1S1'P#62	+	343	1837
Δ pLW1S2'P#4	+	343	1817
Δ pLW1S1'P#10	+	342	1897
Δ pLW1S1'P#26	+	342	1749
Δ pLW1S2'P#13	+	342	1616
Δ pLW1S1'P#34	+	341	1854
Δ pLW1S30sP#40	+	340	1901
Δ pLW1S1'P#65	+	339	1817
Δ pLW1S2'P#49	+	333	1507
Δ pLW1S2'P#5	+	329	1747
Δ pLW1S1'P#1	+	310	1781
Δ pLW1S1'P#38	+	297	1853
Δ pLW1S2'P#9	+	297	1897
Δ pLW1S2'P#46	+	296	1649
Δ pLW1S1'P#5	-	276	1790
Δ pLW1S1'P#15	-	276	1772
Δ pLW1S1'P#20	-	274	1737

Deletion plasmids were assayed for complementation and amount of 5' flanking sequence removed. For the complementation, a + represents growth of TDY2040, transformed with a particular deletion plasmid, on high calcium plates and a - represents failure to grow. The pRS314 end point is the sequence from pRS314 that is adjoined to the *SalI* linker at the junction between pRS314 and *CSG2*. The number refers to the position in the pRS314 sequence as defined by Sikorski and Hieter (1989).

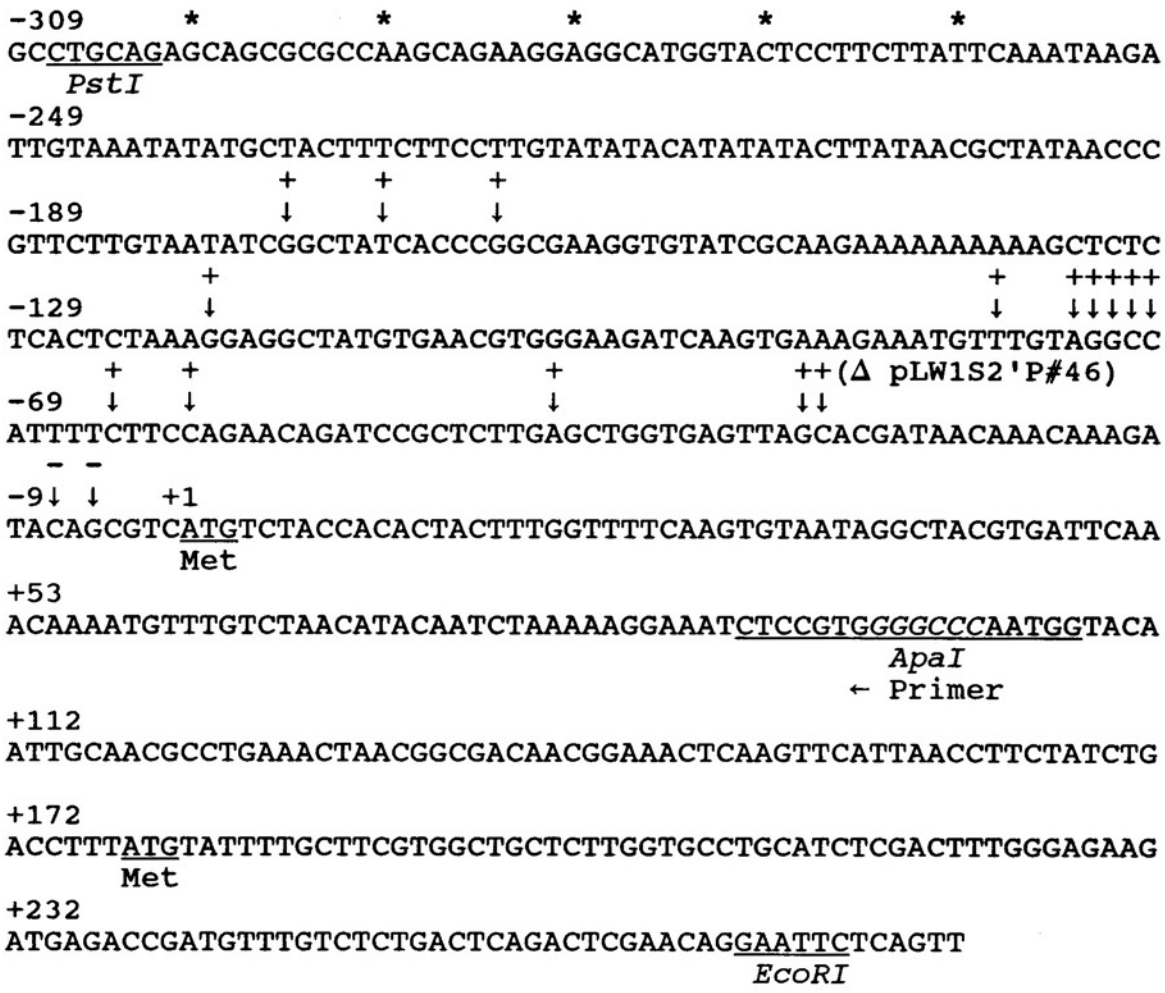

Figure 9. Summarized result from Table 2 shown in the *CSG2* sequence of Figure 2. The plus and minus signs represent complementation or noncomplementation by the deletion mutants. The location of the shortest complementing *CSG2* deletion clone, Δ pLW1S2'P#46, at -27 nucleotides is indicated.

pRS314 sequence removed. From Table 2, Δ pLW1S30sP#40's pRS314 sequence adjoining the *SalI* site is at position 1901 and Δ pLW1S2'P#49's is at position 1507. The positions are as defined by Sikorski and Hieter (1988). Figure 10 shows some of the restriction enzyme positions of pRS314 for orientation relative to the varying end points presented in Table 2. Some positions such as 1897 found in Δ pLW1S30sP#47, Δ pLW1S1'P#10, and Δ pLW1S2'P#9 occurred more often than others; however, the overall occurrence of positions within the 400 nucleotide range appears to be random. The variability of the constant vector sequence adjacent to *CSG2* must have been caused by *in vivo* rearrangements in bacteria, because the deletion fragments were subcloned into a plasmid purified from a single colony.

To determine if the *in vivo* rearrangements of the pRS314 sequence positioned cryptic transcription control elements within functional proximity of the *CSG2* varying 5' flanking end, 4 of the deletion plasmids in Table 2 were selected for further analysis. These deleted *CSG2* mutants were subcloned into vectors with different yeast selective markers and in two orientations so as to be able to eliminate the possibility of cryptic transcription control elements influencing *CSG2* expression. The *SalI* to *KpnI* fragments from Δ pLW1S1'P#1, Δ pLW1S1'P#38, Δ pLW1S2'P#46, and Δ pLW1S1'P#5 were subcloned into pRS314, pRS316, and YCp50. The position of insertion into pRS314 was at the polylinker site downstream of the tryptophan selective marker. In pRS316 and YCp50, the inserts are in the 2 possible orientations with respect to the uracil selective marker. Due to the lack of a suitable *KpnI* site in YCp50 for the subcloning, the *SalI* to *PvuII* fragment from the selected deletion plasmids were actually subcloned into the *SalI* and *NruI* sites of YCp50. Therefore, the YCp50 subclones contain the *SalI* to *KpnI* fragment that has the *CSG2* gene plus about 200 base pairs of pRS314 sequence from the

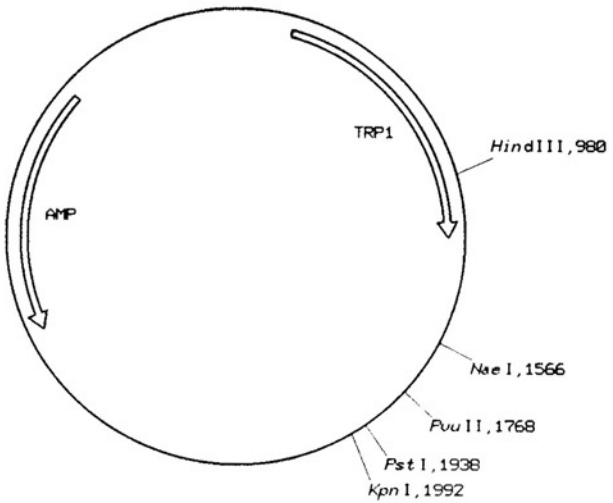

Figure 10. General properties of the vector pRS314. The genes for selective markers, ampicillin resistance and tryptophan metabolism, are indicated. Restriction enzyme sites for position references are indicated as well.

c.v.#4. Figures 11a, 11b, and 11c show the general properties of the *SalI* to *KpnI* subclones in pRS314, pRS316, and YCp50, respectively.

The complementation results of the *SalI* to *KpnI* subclones showed that even less 5' flanking sequence is needed for functional *CSG2* expression than determined in the initial deletion clones. All of the *SalI* to *KpnI* subclones complemented the calcium-sensitive growth phenotype of the *csg2* null mutant including the original noncomplementing deletion plasmid, Δ pLW1S1'P#5. The deleted *CSG2* fragments from this plasmid complemented the *csg2* null mutant phenotype when subcloned into pRS314, pRS316, and YCp50. Only 7 base pairs are present upstream of the *CSG2* AUG codon in Δ pLW1S1'P#5.

DISCUSSION

The result that only about 30 nucleotides in the sequence upstream of the proposed start codon for the *CSG2* gene are required for functional *CSG2* expression was surprising. This finding raises the possibility that a second methionine codon, 59 codons downstream from the first methionine codon, may be the actual start codon for *CSG2*. Within the 177 nucleotides separating the 2 potential start codons, there are 10 additional transcriptional initiation consensus sequences, similar to the ones in Table 1, that could be transcription initiation sites.

From Northern analysis, the size of *CSG2*'s mRNA seems to support the second methionine as the start codon since the size of the mRNA for *CSG2* has been found to be between 1200 to 1400 nucleotides (Chun Zhao, personal communication). Assuming the RNA was not degraded during the purification procedure, it would be unexpectedly small to encode a 410 amino acid *CSG2* gene product beginning from the first methionine, but is sufficiently large to encode a protein beginning at the second

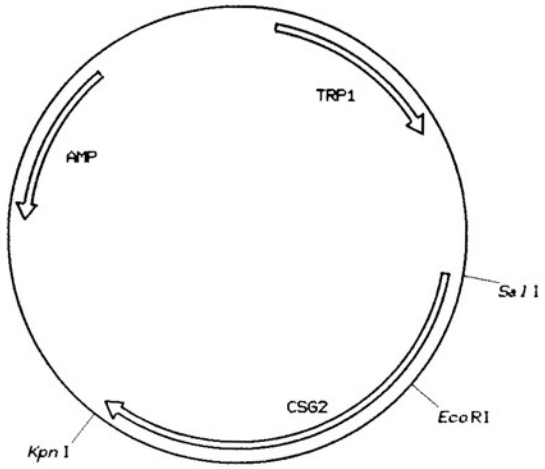

Figure 11a. General properties of the *SalI* to *KpnI* subclones in pRS314. The genes for selective markers, ampicillin resistance and tryptophan metabolism, are indicated. Details are in the text.

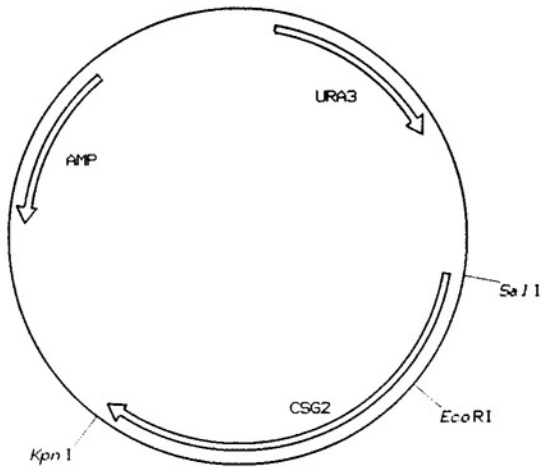

Figure 11b. General properties of the *SalI* to *KpnI* subclones in pRS316. The genes for selective markers, ampicillin resistance and uracil metabolism, are indicated. Details are in the text.

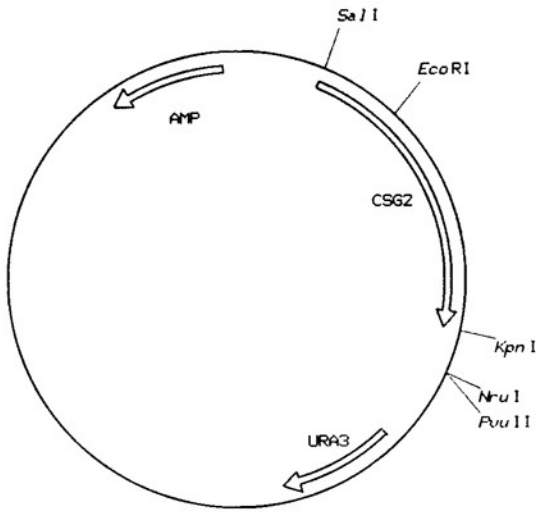

Figure 11c. General properties of the *SalI* to *KpnI* subclones in YCp50. The genes for selective markers, ampicillin resistance and uracil metabolism, are indicated. Details are in the text.

methionine.

Evidence is available that suggests the sequence between the two methionine codons is important for expression of functional *CSG2* (Dunn et al., unpublished data). Thirty-three codons downstream from the first methionine codon and 27 codons upstream of the second methionine codon, is an unique *ApaI* restriction enzyme site in *CSG2*. When a frame-shift mutation is created in *CSG2* by introducing 4 base pairs at the *ApaI* site, the resulting mutant does not complement the calcium-sensitive growth phenotype of the *csg2* null mutant. This result may indicate that the first methionine is indeed the actual translational start site and a functional *CSG2* gene product is not made due to the frame shift. Alternatively, the 4 base pair insertion at *ApaI* may interfere with the expression of *CSG2* by disrupting a transcriptional control element at that site. In either case, more experiments are necessary to determine the exact amount of *CSG2* 5' flanking sequence needed for expression of a functional protein. These experiments include using reverse transcriptase to identify *CSG2*'s transcription start site by primer extension and performing additional deletion analysis with complementation assays for the 5' flanking region of *CSG2*.

Data from primer extension experiments could help determine which methionine is the translational start site of *CSG2*. Cigan and Donahue (1987) compared 131 yeast genes and observed that the first methionine codon from the 5' end of their transcripts is usually the translation start codon. Thus, when the size of the transcript for *CSG2* is determined from primer extension experiments, the amount of *CSG2*'s sequence included in the transcript can be determined and examined to locate the first AUG codon from the 5' end. Once *CSG2*'s translation site is determined, deletion analysis directed at the sequence downstream from it would indicate if downstream transcriptional control

elements exist in *CSG2*. These deletion mutants could then be analyzed for relative transcription levels by quantitative reverse transcription assays or by assays of reporter gene fusions. The results would show if the transcription of *CSG2* is controlled by downstream sequences.

The observation that the sequence adjoining the nested deletions of the 5' flanking sequence of *CSG2* in pRS314 varied from clone to clone was unexpected. As described in earlier sections, the sets of nested deletion fragments were subcloned into c.v.#4 to prevent variability in the vector. Despite these efforts it is possible that *in vivo* rearrangements brought cryptic transcriptional control elements into functional proximity of the *CSG2* start codon. The expression of recombinant plasmids in yeast can be affected by the vector sequence, especially bacterial vector sequence (Marczynski and Jaehning, 1985; Rosenberg et al., 1990). However, we think this is unlikely for the following reasons. First, the variability of the 3' pRS314 sequence appears to be random. Second, there is a clear break between the members of the family of 5' deletion mutants that complement and those that do not. Therefore, it seems unlikely that the variability in the position of fusion of pRS314 to *CSG2* is influencing expression of *CSG2*.

The complementation results for the *SalI* to *KpnI* subclones suggest an even shorter 5' flanking sequence may be sufficient for functional *CSG2* expression, since the *csg2* null mutant's phenotype is complemented by the *SalI* to *KpnI* subclones of the deletion plasmid, Δ pLW1S1'P#5. The original deletion for Δ pLW1S1'P#5 in c.v.#4 did not complement. There are two more noncomplementing *CSG2* deletion mutants in the clear break between the complementing and noncomplementing plasmids in Table 2. Complementation assays and primer extension analysis could be performed with the *SalI*

to *KpnI* subclones of these *CSG2* deletion mutants to help clarify results obtained with Δ pLW1S1'P#5. Combined results from these experiments would provide further insight into the transcriptional control of *CSG2* by its 5' flanking sequence.

In summary, 3 interpretations are offered to explain how such a short 5' flanking sequence for *CSG2* can complement the *csg2* null phenotype. First, the start site for the *CSG2* gene may not be the proposed methionine codon but the methionine 177 nucleotides downstream from it. Therefore, more of the 5' flanking sequence needs to be removed before *CSG2* expression will be affected. The second possibility is that the expression of *CSG2* may be controlled by downstream transcriptional control elements which were not affected by deletions at the 5' end of the gene. Lastly, *CSG2* may indeed require only a short 5' flanking sequence for expression of its functional protein.

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